In re Application of: Shabat et al
Serial No.: 10/525,951
Filed: February 28, 2005
Office Action Mailing Date: December 10, 2008

Examiner: Trevor M. Love
Group Art Unit: 1611
Attorney Docket: 29195

REMARKS

Reconsideration of the above-identified application in view of the amendments above and the remarks following is respectfully requested.

Claims 1-175 are in this Application. Claims 103-160 have been withdrawn from consideration for being drawn to a non-elected invention. Claims 1-102 and 161-175 have been examined on the merits. Claims 1-102 and 161-175 have been rejected. Claim 83 has been objected to. Claims 35-53 and 84-101 have been canceled herewith. Claims 1, 15, 16, 22, 69, 70, 76, 83, 172 and 174 have been amended herewith. New claims 176-205 have been added herewith.

Election/Restriction

The Examiner has stated that upon reconsideration Groups I, II, III and XII should be rejoined.

The Examiner has further stated that upon further reconsideration, Groups V, VI and VII should be rejoined.

The Examiner has further stated that upon reconsideration the species election was improper and has been withdrawn.

Accordingly, the Examiner has stated that claims 1-102 and 161-175 are currently under consideration, in their generic context.

Claim Objection

The Examiner has stated that claim 83 is objected to since it ends with two periods and that appropriate correction is required.

Claim 83 has been amended accordingly, to thereby overcome the Examiner's objection.

35 U.S.C. § 112, First paragraph Rejection

The Examiner has stated that claims 16-23, 35-53, 70-77, 84-101 and 162-175 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written

description requirement. The Examiner's rejection is respectfully traversed. Claims 35-53 and 84-101 have been canceled, thereby rendering moot the Examiner's rejection with respect thereto. Claims 16, 22, 70, 76, 172 and 174 have been amended.

Specifically, the Examiner has stated that the MPEP states that for a generic claim the genus can be adequately described if the disclosure presents a sufficient number of representative species that encompass the genus (MPEP § 2163) and that if the genus has a substantial variance, the disclosure must describe a sufficient variety of species to reflect the variation within that genus. The Examiner has further stated that in the Guidelines for Examination of Patent Applications Under 35 USC 112, first, written description requirement, in accordance with MPEP § 2163, it is specifically stated that for each claim drawn to a genus, the written description requirement may be satisfied through sufficient description of a representative number of species by (a) actual reduction to practice; (b) reduction to drawings or structural chemical formulas; (c) disclosure of relevant, identifying characteristics by functional characteristics coupled with a known disclosed correlation between function and structure.

The Examiner has carried out a comparison of the scope of the claims with the scope of the description, and has stated that (i) substantial structural variation exists in the genus/subgenera embraced by claims 16-23, 35-53, 70-77, 84-101, 162-175; (ii) disclosure of species supporting genus is limited to compounds reduced to practice, which scope is not commensurate with the scope of genus/subgenera claimed; (iii) common structural attributes of the genus/subgenera, combined with a correlation between structure and function, is neither disclosed in the instant application nor commonly known in the art.

The Examiner has concluded by stating that the specification fails to provide adequate written description for the genus of compounds claimed and does not reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, has possession of the entire scope of the claims invention.

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In response, Applicant wishes to direct the Examiner's attention to the description spanning from page 18, line 17 to page 21, line 11, where the structural features of the claimed self-immolative chemical linkers that are required for its desired function in the claimed self-immolative dendrimer are described, and particularly to the following passages in the instant application:

Page 18, lines 25-27, where it is recited:

"*As is described hereinabove, the self-immolative chemical linker of the present invention is selected such that it undergoes a sequence of self-immolative reactions upon cleavage of the trigger unit.*

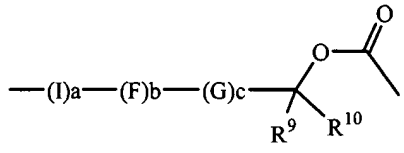
As is known in the art, self-immolative reactions typically involve electronic cascade self-elimination and therefore self-immolative systems typically include electronic cascade units which self-eliminate through, for example, linear or cyclic 1,4-elimination, 1,6-elimination, etc. Such electronic cascade units are widely described in the art (see, for example, WO 02/083180)."

Page 19, lines 4-7, where it is recited:

"*Such chemical linkers are preferably based on a multifunctional aromatic unit which can be linked to both the trigger unit and to two or more tail units or other chemical linkers and can further be subjected to electronic cascade self-elimination.*"

And to the paragraph bridging pages 20-21, where it is recited:

"*The –(I)a-(F)b-(G)c- unit, if present, is a linear electronic cascade unit that is conjugated to the aromatic system of the basic unit and thereby directly participate in the self-immolative reactions sequence, whereas the carboxy unit –O-(C=O)- enables the release of the linkers/tail units attached thereto via a decarboxylation, which takes place at the end of the self-immolation sequence. The presence of two or more such*



groups as substituents of the aromatic system enables the occurrence of more than one self-immolative reactions sequence at a time.

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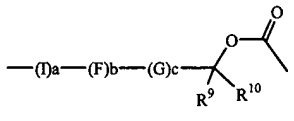
The aromatic system, while being capable to undergo various rearrangements, further enables such occurrence".

It is therefore submitted that the instant specification clearly describes the structural features required for the function of the claimed self-immolative chemical linkers, and further explains the mode of action of these self-immolative chemical linkers.

Accordingly, the presence of an aromatic base unit, as reflected in the claimed Formulae Ia and Ib, and of a linear electronic cascade unit attached thereto, as reflected by the claimed  group, and their related function, are clearly described.

By being a self-immolative system that comprises the above-described structural elements required for the self-immolation, all other optional substituents are not essential for the activity of the claimed self-immolative dendrimers.

It is further submitted that while, as described in the instant specification and shown hereinabove, the basic structural features of the claimed self-immolative chemical linkers are selected so as to impart the linker the capability to self-immolate via electronic cascade self-elimination, the electronic cascade units that form the claimed self-immolative chemical linkers are known in the art in the context of self-elimination.

The Examiner's attention is directed in this regard, for example, to WO 02/083180 (De Groot), which is cited both in the instant application and by the Examiner, and to the corresponding U.S. Patent No. 7,223,837. De Groot teaches self-immolative systems which have basic structural elements identical to those of the claimed self-immolative linkers. See, for example, the description spanning from page 3, line 27, to page 5, line 31, in De Groot, and particularly the passage on page 5 relating the non-substantial effect of the substituents on the function of the system described therein, and the paragraph bridging pages 9 and 10 therein. The structural elements of the claimed self-immolative linkers that are different from those described

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in De Groot are delineated hereinbelow, Re: USC 103 Rejections. However, the structural elements that are distinct from those described in De Groot merely allow the formation of a dendrimeric structure, and do not affect the self-elimination reactions that the claimed self-immolative linkers are capable of undergoing.

It is therefore submitted that the instant specification describes all the relevant, identifying characteristics of the claimed self-immolative linkers, by functional characteristics, coupled with a known disclosed correlation between function and structure, and hence meets the written description requirement.

The Examiner's attention is further directed in this regard to a publication of which one the present inventors, Doron Shabat, is a co-author: Sagi et al., Bioorganic & Medicinal Chemistry, 15 (2007), 3720–3727, a copy of which is enclosed herewith. This publication describes the preparation and practice of self-immolative dendrimers that contain self-immolative chemical linkers encompassed by the claimed Formula Ib, in which V is –NH-. Thus, it is further shown that as long as the self-immolative linker includes an aromatic moiety that is substituted by a moiety that enables self-elimination via electronic cascade reactions, as in the claimed self-immolative chemical linkers and the corresponding description thereof, the desired function thereof is obtained.

Applicant wishes to further direct the Examiner's attention to the description spanning from page 27, line 9 to page 30, line 26, where the structural elements of the claimed self-immolative spacer that are required for its desired function in the claimed self-immolative dendrimer are described, and particularly to the following passages in the instant application:

Page 28, lines 25-27, where it is recited:

"Being selected as self-immolative, the spacer of the present invention participates in the self-immolative reactions sequence of the SIDs of the present invention".

And page 30, lines 3-6, where it is recited:

"The spacers presented by Formulas IIa, IIb, IIc and IId therefore belong to the known □amino aminocarbonyl cyclization spacers, which undergo self-elimination via a cyclization process (as is exemplified, for example, in Figures 3, 7 and 22), so as to form urea derivatives".

It is therefore submitted that the instant specification clearly describes the structural elements required for the function of the claimed self-immolative spacers, and further explains the mode of action of these self-immolative spacers.

It is further submitted that while, as described in the instant specification and shown hereinabove, the basic structural features of the claimed self-immolative spacers are selected such the spacer can participate in the self-immolative reaction sequence of the claimed dendrimers, the electronic cascade units that form the claimed self-immolative spacers are well-known in the art in the context of self-elimination.

The Examiner's attention is directed in this regard, for example, to WO 02/083180 (De Groot), which is cited both in the instant application and by the Examiner, and to the corresponding U.S. Patent No. 7,223,837. De Groot teaches self-immolative systems which include self-immolative units that have basic structural elements identical to those of the claimed self-immolative spacers. See, for example, the description spanning from page 6, line 21, to page 7, line 24.

It is therefore submitted that the instant specification describes all the relevant, identifying characteristics of the claimed self-immolative spacers, by functional characteristics coupled with a known disclosed correlation between function and structure.

Accordingly, it is submitted that claims 16-23, 70-77, and 162-175 comply with the written description requirement.

35 U.S.C. § 112, Second paragraph Rejections

The Examiner has stated that claims 15-18, 22-23, 31, 45-48, 52-53, 69-72, 76-77, 83, 93-96, 100-101, and 171-175 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim

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the subject matter which Applicant regards as the invention. The Examiner's rejections are respectfully traversed. Claims 45-48, 52-53, 93-96 and 100-101 have been canceled. Claim 15, 16, 22, 69, 70, 76, 172 and 174 have been amended.

In one particular, the Examiner has stated that claims 15-18, 22-23,45-48, 52-53, 69-72, 76-77, 93-96, 100-101, and 172-175 recite the limitation "R^9-R^{10}" or "R^{27}-R^{28}" in the description of the structures therewithin, and that there is insufficient antecedent basis for this limitation in the claim since the definition of "R^9-R^{10}" is not provided until dependent claims 19, 49, 73, and 97, and the definition of "R^{27}-R^{28}" is not provided. The Examiner has stated that this renders claims 15-18, 22-23, 45-48, 52-53, 69-72, 76-77, 93-96, 100-101, and 172-175 indefinite for failing to clearly set forth the metes and bounds of the instant claims.

Applicant respectfully notes that the limitation "R^9-R^{10}" is not recited in claim 15 but rather in claim 16.

Applicant further respectfully notes that the definition of these variables was unintentionally omitted from the claims.

Claim 16 has been amended so as include a definition of the variables R^9 and R^{10}, so as to correct this unintentional mistake.

Support for this amendment is found, for example, on page 21, lines 16-21, where it is recited:

"*Other ring substituents, as well as the other substituents in Formulas Ia and Ib, R^6, R^7, R^8, **R^9, R^{10}**, R^{11} and R^{12}, can be hydrogen, alkyl, aryl, cycloalkyl, heterocycloalkyl, heteroaryl, alkoxy, hydroxy, thiohydroxy, thioalkoxy, aryloxy, thioaryloxy, amino, nitro, halo, trihalomethyl, cyano, C-amido, N-amido, cyclic alkylamino, imidazolyl, alkylpiperazinyl, morpholino, tetrazole, carboxy, carboxylate, sulfoxy, sulfonate, sulfonyl, sulfixy, sulfinate, sulfinyl, phosphonooxy or phosphate*". (emphasis added).

Claims 70 and 172 have been similarly amended.

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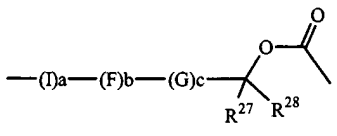
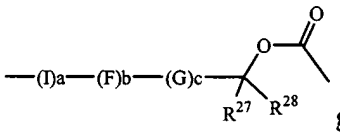
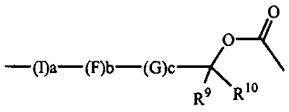
Accordingly, claim 22 has been amended so as to include a definition of the variables R^{27} and R^{28} present in the  group.

Claims 76 and 174 have been similarly amended.

Applicant respectfully notes that the definition of these variables was unintentionally omitted from the claims.

Applicant submits that in view of the similarity between the structure and function of the  group in claims 22, 76 and 174 and the  group in claims 16, 70 and 172, it is clear that the variables R^{27} and R^{28} should be defined as the variables R^9 and R^{10} are.

Claims 45-48, 52-53, 93-96 and 100-101 have been canceled, thereby rendering moot the Examiner's rejection with respect thereto.

Applicant therefore believes to have overcome the Examiner's rejection in this regard.

In another particular, the Examiner has stated that claims 15, 31, 45, 69, 83, 93, and 171 recite "a single absorber agent" (emphasis added by the Examiner) and that this appears to be an obvious typo that should be corrected.

Claims 45 and 93 have been canceled, thereby rendering moot the Examiner's rejection with respect thereto.

Claims 15, 69 and 171 have been amended so as to correct this obvious typographical error and to recite "a signal absorber agent" (emphasis added).

It is noted that claims 31 and 83 as originally filed correctly recite "a signal absorber agent" (emphasis added) and hence have not been amended.

Applicant believes to have overcome the Examiner's rejection in this regard.

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35 U.S.C. § 103 Rejections

The Examiner has stated that claims 1-102 and 161-175 are rejected under 35 U.S.C. 103(a) as being unpatentable over Baker et al. (U.S. Patent No. 6,471,968) in view of De Groot et al. (WO 02/083180) in further view of Greenwald et al (J. Med. Chem.). The Examiner's rejections are respectfully traversed. Claims 35-53 and 84-101 have been canceled. Claims 1, 15, 16, 22, 69, 70, 76, 83, 172 and 174 have been amended.

Specifically, the Examiner has stated that Baker teaches a therapeutic and diagnostic multifunctional system comprising a dendrimer that uses imaging and triggering release of a therapeutic or diagnostic material, that there can be multiple therapeutic agents attached to the dendrimer that act synergistically, that the therapeutic agent can be activated upon release, and said release can be enzymatic cleavage or photo-cleavage, that the composition can comprise a pharmaceutically acceptable carrier, and that the active agent can be an anti-microbial agent, and has further stated that all of the above, upon combination with the aforementioned references, read on instant claims 15, 9-11, 26-29, 31, 33, 34, 41, 45, 64, 65, 69, 83, 93, 102 and 171.

The Examiner has continued stating that Baker fails to directly disclose that the dendrimer is self-immolative, and further fails to disclose the exact structure of, for instance, the linker or spacer that is enzymatically cleaved.

The Examiner has referred to De Groot, and has stated that De Groot teaches a self-immolative compound comprising a cleavable trigger unit, at least one end unit, self-immolative linkers, and self-immolative spacers, wherein upon cleavage of said trigger unit, the composition self-immolates, and further teaches that the end units comprise anti-cancer agents such as daunorubicin, that the cleavable trigger unit is disclosed as being enzymatically cleaved, and that the end units are taught as being either therapeutically effective active agents, or diagnostic agents, and that the compound can be mixed with a pharmaceutically acceptable carrier for purposes of delivery, and has further stated that all of the above, upon combination with the above

identified references, read on instant claims 1-8, 12, 35, 36, 38-40, 42, 57-63, 66, 80, 82, 87-90, 161, 162, 165-168.

The Examiner has continued stating that De Groot further discloses self-immolative linkers and spacers which, upon combination with above-identified references, read on instant claims 16-23, 46-53, 70-77, 94-101, and 172-175.

The Examiner has further stated that Greenwald teaches a tripartite drug comprising a trigger, linker, and drug, in which the trigger is enzymatically cleaved and the drug can be either para-aniline or daunorubicin, and further discloses that the linker can have the branch that comprises the drug in different locations.

The Examiner has stated that it would have been obvious to one of ordinary skill in the art at the time the invention was made to combine the teachings of Baker, De Groot, and Greenwald, so as to allow the composition of Baker to experience release of the therapeutic agents and diagnostic agents upon a single self-immolative triggered event. The Examiner has further stated that by modifying the teachings of Baker with the teachings of De Groot and Greenwald, the composition of Baker would be able to release an increased number of drugs (tail units) upon a single natural or manmade trigger, and that the single trigger would be able to have a smaller concentration in the self-immolative system than if each cleavage was dependent on individual actions of the trigger. The Examiner has continued stating that there would be a reasonable expectation in the success of the combination since all three compositions teach enzymatic cleavage of daunorubicin, and all three compositions are designed as therapeutic/diagnostic compositions designed for cancer treatment/diagnosis.

The Examiner has further stated that instant claims 55 and 56, and 24-25, 37, 78-79, 85-86, and 164, are also rendered obvious.

In response, Applicant respectfully notes the following:

The instant application, in some embodiments thereof, is of self-immolative dendrimers that include a cleavable unit as the core, a plurality of self-immolative units that extend outwardly therefrom and a plurality of functional group as the tail

units. The self-immolative dendrimers described and claimed in the instant are capable of releasing all of the functional moieties simultaneously, as a response to a single event.

As widely discussed in the instant application, other known applications which utilize the high-group functionality of dendrimers for amplifying the effect of the functional tail groups involve the release of each of the functional tail groups upon an associated cleavage event. Therefore, the efficacy of such dendrimers is limited, as they require a plurality of events to achieve substantial amplification of tail units release.

The present invention therefore provides a solution to a long-felt need for more efficient compounds that are capable of simultaneously releasing a plurality of functional moieties.

The self-immolative dendrimers taught by the instant application were designed by combining the unique structural properties and synthetic routes of dendrimers and technologies that involve self-immolative systems.

As demonstrated in the instant application, it was found that various dendrimers, designed per the above-described rationale, are both synthesizable and are indeed capable of releasing a plurality of functional tail units upon a single cleavage event. More specifically, it was found that subjecting such dendrimers to conditions that prompt cleavage of the core, triggers a sequence of reactions that results in self-immolation of the dendrimer and thus leads to a spontaneous release of all the tail units upon a single event.

Applicant contends that the design of such self-immolative dendrimers has not been suggested nor described before the invention was made, neither in the general art nor in the documents cited by the Examiner.

Baker teaches nanodevices comprised of a dendrimers complex of two or more dendrimers that ate physically attached to one another, wherein each dendrimer can possess a different agent, either as its tail units or as entrapped molecules.

The tail units in the dendrimers taught in Baker are each released upon cleavage of the bonds through which they are attached to the dendrimeric structure.

Baker in therefore completely silent with respect to the design and practice of self-immolative dendrimers.

Moreover, while Baker can be regarded as addressing the issue of releasing various functional groups by providing a complex of dendrimers, each capable of releasing different functional groups, Baker does not even remotely suggest that such a release can be performed by manipulating the structural elements of common dendrimers.

De Groot and Greenwald et al. both teach compounds that are capable of releasing one functional molecule upon a cleavage event. The compounds taught in these documents were designed to include a self-eliminating moiety and an enzymatically-cleavable moiety.

However, while devising the compounds taught therein, both De Groot and Greenwald et al. aimed at providing prodrugs with improved activation characteristics, extended half-lives, improved solubility and other parameters (see, for example, the Abstract in Greenwald et al., and from page 2, line 7, to page 8, line 16 in De Groot).

Accordingly, both De Groot and Greenwald et al. are completely silent with respect to the use of the self-eliminating moieties taught therein for devising self-immolative dendrimers that simultaneously release all of their tail units upon a single cleavage event. Moreover, De Groot and Greenwald et al. both fail to address the long-felt need for such dendrimers and hence provide no motivation to combine the methodologies taught therein with the unique structural characteristics of dendrimers.

It is therefore submitted that combining the unique structural properties and synthetic routes of dendrimers and technologies that involve self-immolative systems, so as to design the claimed self-immolative dendrimers, which simultaneously release all of their tail units upon a single cleavage event, could not have been made in view of the teachings of the cited documents without an inventive activity.

Moreover, it is further submitted that designing such self-immolative dendrimers which are both synthesizable and indeed release a plurality of functional tail units upon a single cleavage event, could not have been made in view of the teachings of the cited documents without an inventive activity.

In this regard, it is respectfully submitted that, as is well-recognized in the chemistry field, let alone the medicinal chemistry field, a successful design that combines different structural elements into a single compound is never trivial, as synthetic chemistry depends on many factors such as, for example, steric and electronic intramolecular and intermolecular interactions, thermodynamic and kinetic considerations, and the like.

Moreover, such a successful design which is both synthesizable and exhibits the desired function (herein, simultaneous release of all tail units upon a single cleavage event) is even less trivial.

It is therefore submitted that even if the cited documents teach, on one hand, multifunctional dendrimeric system (Baker), and on the other end, compounds that release a drug upon self-elimination (De Groot and Greenwald et al.), successfully combining these features could not have been made without an inventive activity.

It is further noted that while devising the claimed self-immolative dendrimers, the present inventors have considered the methodologies known in the art, including, for example, the methodologies taught in De Groot.

However, while utilizing these methodologies in the context of dendrimers, modifications of these methodologies were made. Thus, for example, while De Groot teaches spacers that self-eliminate through a simple, single 1,(4+2n)-elimination (see, for example, Figures 2-5 therein), embodiments of the present invention are of self-immolative linkers that can undergo double or even triple self-eliminating rearrangements (see, for example, Example 1 and Figure 3 of the instant application).

As noted hereinabove, successfully designing such moieties cannot be made without an inventive activity.

It is therefore submitted that while the Examiner's statement that "*by modifying the teachings of Baker with the teachings of De Groot and Greenwald, the composition of Baker would be able to release an increased number of drugs (tail units) upon a single natural or manmade trigger, and that the single trigger would be able to have a smaller concentration in the self-immolative system than if each cleavage was dependent on individual actions of the trigger*" maybe correct in retrospect, designing and performing such a modification cannot be simply made without an inventive activity, as chemistry is not mathematics. It is submitted, however, that the Examiner's statement further reflects the need for the inventive activity presented in the instant application.

Applicants submit that the examiner's conclusion of obviousness includes knowledge gleaned *only* from Applicant's disclosure, and is therefore based on improper hindsight reasoning, since it does not take into account only knowledge which was within the level of ordinary skill in the art at the time the claimed invention was made.

It is further submitted that the Examiner's statement that "*there would be a reasonable expectation in the success of the combination since all three compositions teach enzymatic cleavage of daunorubicin, and all three compositions are designed as therapeutic/diagnostic compositions designed for cancer treatment/diagnosis*" is completely false, since the function exhibited by the claimed self-immolative dendrimers do not depend solely on the presence of an enzymatically-cleavable trigger unit and/or the release of therapeutically active agents and/or diagnostic agents, but is mainly attributed to the presence of the claimed self-immolative chemical linkers and its self-immolation compatibility with the trigger unit the releasable tail unit and the optional spacer.

Thus, it is submitted that the claimed invention is not rendered unpatentable over Baker in view of De Groot and Greenwald et al., and is therefore allowable.

Notwithstanding the above, Applicant has chosen, in order to more clearly distinct the claimed invention from the cited art, to amend claim 1 so as to recite that

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"... *upon cleavage of said trigger unit, said at least one self-immolative chemical linker self-immolates, thereby* ***simultaneously*** *releasing* ***all of*** *said tail units*" (emphasis added).

Additional Amendments and New claims

In view of the Examiner's statement regarding rejoining Groups I, II, III and XII, Applicant has chosen to cancel claims 35-53 for reading on subject matter already included within claims 162-175.

Applicant has further chosen to cancel claims 84-101.

Applicant has further chosen to add new claim 176, pertaining to a pharmaceutical composition that comprises a self-immolative claim as defined in previously presented claim 167.

The subject matter of new claim 176 corresponds to the subject matter of now canceled claim 84.

Applicant has further chosen to add new claims 177-205, pertaining to self-immolative dendrimers and compositions comprising same, in which the self-immolative linkers are defined as having Formula Ia and/or Ib.

Support for the subject matter of new independent claim 177, and the claims depending therefrom, is found, for example, in claim 16, and claims depending therefrom, of the application as originally filed.

It is therefore submitted that no new subject matter is added herewith.

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In view of the above amendments and remarks it is respectfully submitted that amended claim 1, claims 1-14, amended claims 15 and 16, claims 17-21, amended claim 22, claims 23-34, claims 54-68, amended claims 69 and 70, claims 71-75, amended claim 76, claims 77-82, amended claim 83, claim 102, claims 161-170, amended claim172, claim 173, amended claim 174, claims 175 and new claims 176-205 are now in condition for allowance. A prompt notice of allowance is respectfully and earnestly solicited.

Respectfully submitted,

Martin D. Moynihan
Registration No. 40,338

Date: May 11, 2009

Enclosures:

- ☐ Petition for Extension (Two Months)
- ☐ Additional Claims Transmittal Fee
- ☐ Reference: Sagi et al

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Remarkable drug-release enhancement with an elimination-based AB_3 self-immolative dendritic amplifier

Amit Sagi,[a] Ehud Segal,[b] Ronit Satchi-Fainaro[b,*] and Doron Shabat[a,*]

[a]*Department of Organic Chemistry, School of Chemistry, Raymond and Beverly Sackler Faculty of Exact Sciences, Tel Aviv University, Tel Aviv 69978, Israel*
[b]*Department of Physiology and Pharmacology, Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978, Israel*

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Abstract—Self-immolative dendritic prodrugs, activated through a single catalytic reaction by a specific enzyme, could offer significant advantages in inhibition of tumor growth relative to monomeric prodrug, especially if the targeted or secreted enzyme exists at relatively low levels in the malignant tissue. We have designed and synthesized new AB_3 self-immolative dendritic prodrug system that releases three active drugs by a single cleavage of the enzyme penicillin-G-amidase. The cleavage signal is transferred from the dendron focal point to its periphery through fast elimination reactions and the design leads to three-fold signal amplification. In cell-growth inhibition assays, the elimination-based AB_3 self-immolative dendritic prodrug was significantly more effective than a cyclization-based AB_3 dendritic prodrug.

1. Introduction

Self-immolative dendrimers are novel class of molecules that can amplify a single cleavage event received at the focal point into multiple release of tail groups at the periphery.[1–3] These dendrimers can be used for the construction of dendritic prodrugs, if a specific trigger is attached to the focal point and drug molecules are linked to the periphery.[4,5] Self-immolative dendritic prodrugs, activated through a single catalytic reaction by a specific enzyme, could offer significant advantages in inhibition of tumor growth relative to monomeric prodrug, especially if the targeted or secreted enzyme exists at relatively low levels in the malignant tissue. We have shown that single-triggered dendritic prodrugs significantly inhibit tumoral cell growth compared to classic monomeric prodrugs.[6] The dendritic platform was also used for the synthesis of a single-triggered hetero-dimeric prodrug derivatized with the anticancer agents doxorubicin and camptothecin.[7] This prodrug made it possible to release two different chemotherapeutic drugs simultaneously at the same location. In another report, we designed and synthesized fully biodegradable dendrimers that disassemble through multi-enzymatic triggering followed by self-immolative chain fragmentation.[8] A practical application for such multi-triggered self-immolative dendrons was recently demonstrated by the concept of prodrug activation through a molecular OR logic trigger.[9,10]

Rapid release of tail-drug units from the dendritic platform is essential in order to achieve maximal drug concentration at a specific location. Therefore, self-immolative dendrons with a fast disassembly mechanism should have a significant advantage in a dendritic prodrug system.[11] Here we report the design and synthesis of a fast AB_3 self-immolative dendritic prodrug system, activated through a single enzymatic cleavage by penicillin-G-amidase (PGA).[12]

2. Results and discussion

Recently, we reported the synthesis and activation of dendritic prodrug similar to **1**.[6] This prodrug releases three molecules of active drug upon single cleavage by

Abbreviations: ACN, acetonitrile; DCM, dichloromethane; DIPEA, diisopropylethyleneamine; DMF, dimethyl formamide; DMAP, dimethylaminopyridine; EtOAc, ethylacetate; Et_3N, triethylamine; Hex, hexane; MeOH, methanol; Mel, melphalan; PNP, *p*-nitrophenol; PNA, *p*-nitroaniline; TBSCl, *tert*-butyldimethylsillyl chloride; THF, tetrahydrofuran; Trp, tryptophan.

Keywords: Prodrug; Dendrimer; Enzyme; Self-immolative.

* Corresponding authors. Tel.: +972 (0) 3 640 8340; fax: +972 (0) 3 640 9293; e-mail: chdoron@post.tau.ac.il

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PGA (Fig. 1). The disassembly pathway is initiated by removal of phenylacetic acid, elimination of azaquinone-methide, and decarboxylation to generate amine-intermediate **1a**. The latter cyclizes to release dimethylurea derivative and phenol **1c**, which rapidly undergoes triple elimination to release the three drug units. The rate-determining step was found to be the cyclization of **1a–1c**.

In order to enhance the release-rate of the peripheral drug units, we sought to design a system that would disassemble without the slow cyclization step. Taking into consideration this objective, we designed AB_3-dendritic molecule **2**. The first step in the reaction is catalytic cleavage of phenylacetic acid by PGA,[13] followed by elimination of azaquinone-methide and decarboxylation to release amine-intermediate **2a**. This amine intermediate further disassembles through triple elimination to release the three drug units (Fig. 2). The disassembly of this molecule after the enzymatic cleavage is solely based on elimination reactions and therefore is expected to occur very rapidly.

Although molecule like **2a** was reported before,[2] there is no published procedure for its synthesis. We developed a simple and efficient new synthetic pathway for dendritic compounds like **2**, reported here. To compare the disassembly rate of the above dendritic systems, we synthesized AB_3 molecules **3** and **4** (Fig. 3). Both molecules are designed for activation by PGA and have three units of tryptophan as a model drug. Tryptophan was used for initial evaluation as it contains a strong UV chromophore, allowing us to monitor the disassembly reaction.

The synthesis of compounds **3** and **4** was performed as presented in Figures 4 and 5. Compound **5** was

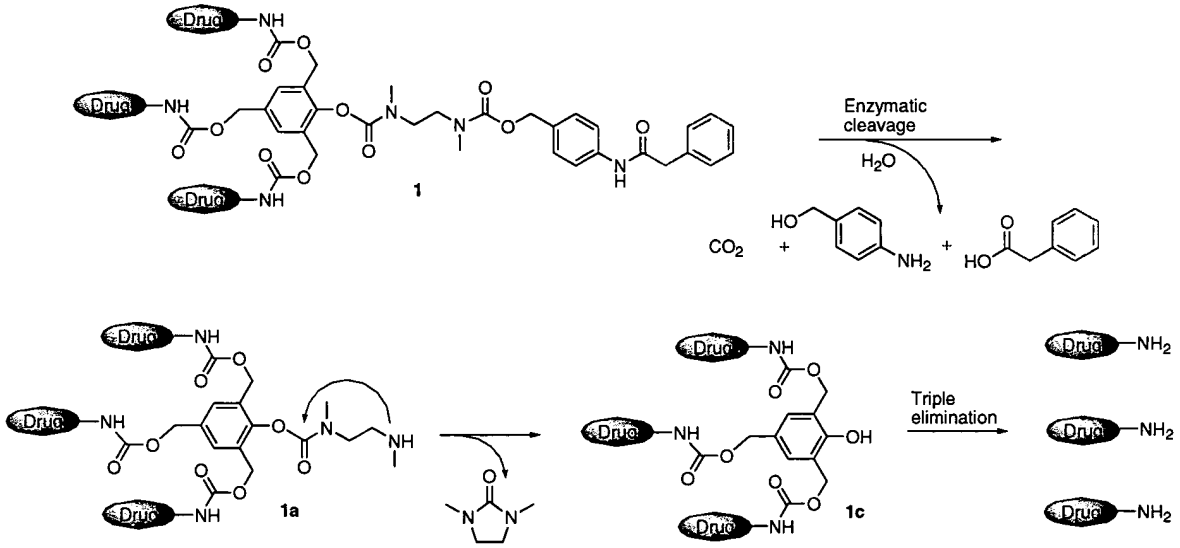

Figure 1. Disassembly mechanism of AB_3 self-immolative dendritic molecule **1**.

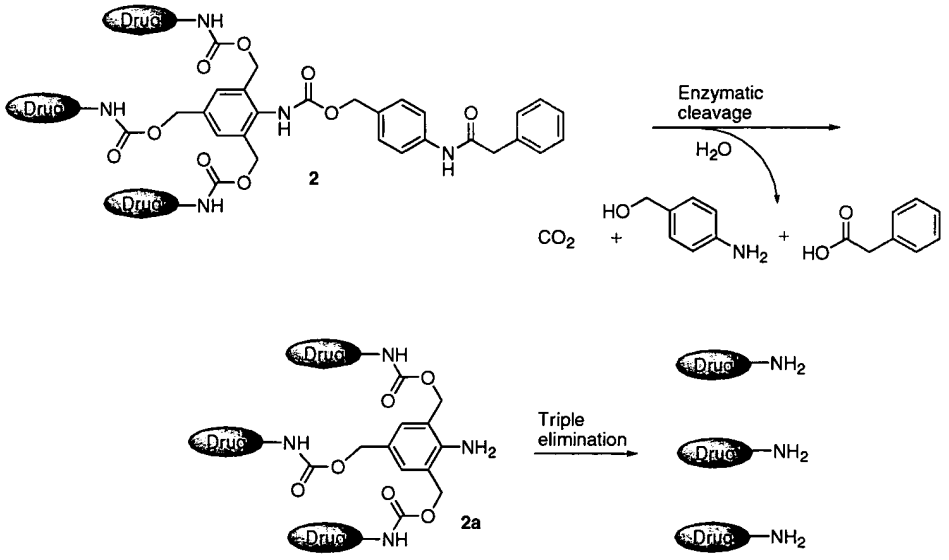

Figure 2. Disassembly mechanism of AB_3 self-immolative dendritic molecule **2**.

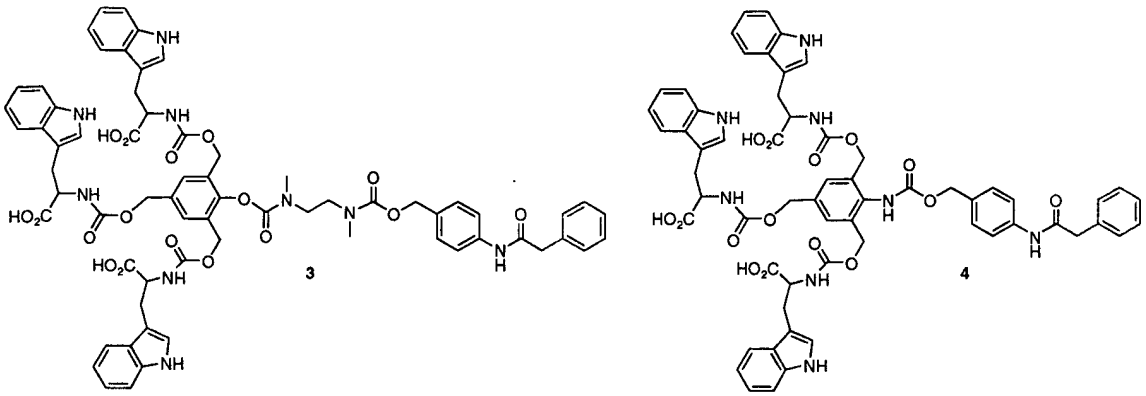

Figure 3. Chemical structures of AB_3 self-immolative dendritic molecules with tryptophan tail units and a trigger that is activated by PGA.

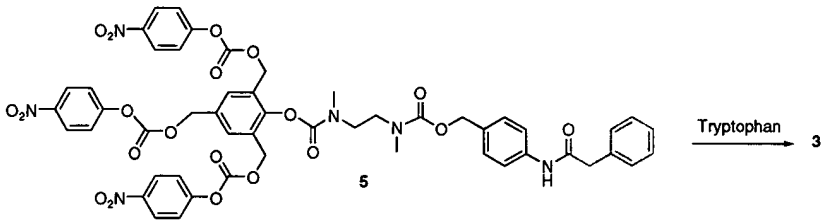

Figure 4. Chemical synthesis of dendritic molecule **3**.

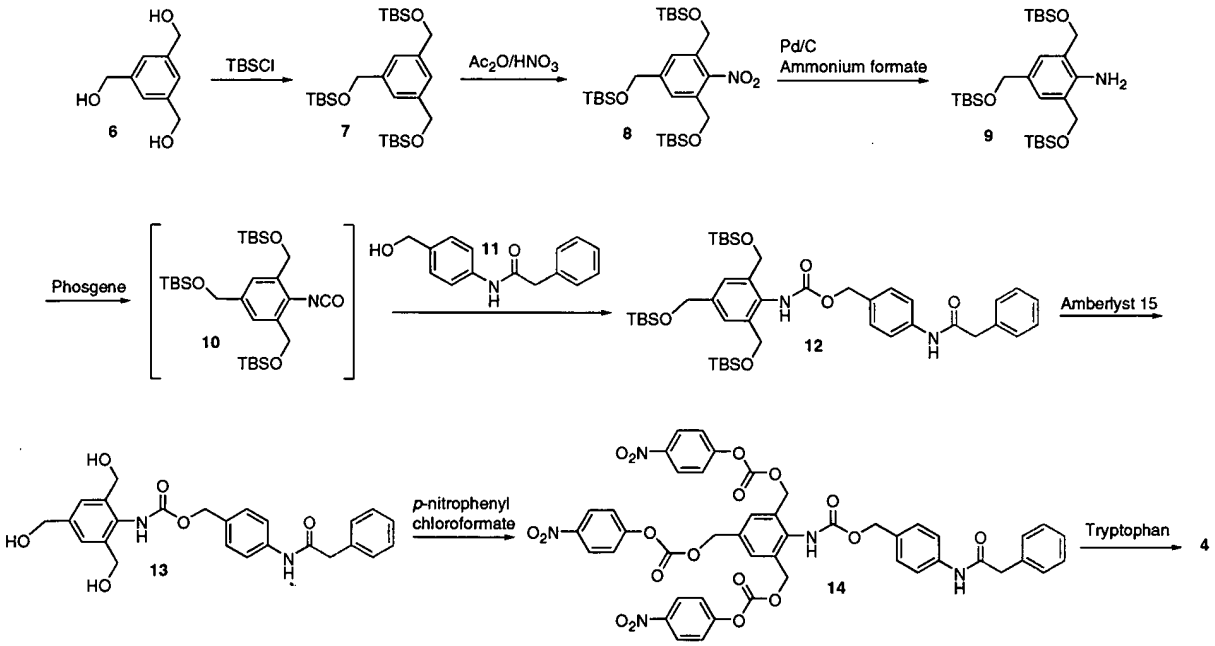

Figure 5. Chemical synthesis of dendritic molecule **4**.

synthesized as previously described.[6,14] Three equivalents of tryptophan was reacted with tricarbonate **5** to generate dendritic molecule **3** (Fig. 4).

Dendritic molecule **4** was prepared starting from tri(hydroxymethyl)-1,3,5-benzene **6**. Thus, triple protection with *tert*-butyldimethylsilyl chloride of the hydroxy groups of **6** gave compound **7** and reaction with nitric acid and acetic anhydride afforded compound **8**. The latter was reduced with palladium on carbon and ammonium formate to give amine **9**. Reaction of phosgene with **9** in situ generated isocyanate **10**, which was immediately reacted with alcohol **11** to give compound **12**. Deprotection of the *tert*-butyldimethylsilyl groups of **12** with amberlyst-15 afforded triol **13**, which was activated with 4-nitrophenyl-chloroformate to give tricarbonate

14. The latter was reacted with three equivalents of tryptophan to generate AB_3 dendritic molecule **4**.

The disassembly reactions of dendritic molecules **3** and **4** were evaluated in phosphate-buffered saline (PBS, pH 7.4) in presence and absence of PGA. The release of tryptophan was monitored by a reverse-phase HPLC at a wavelength of 320 nm. The results are presented in Figures 6 and 7. No disassembly of either system was observed in the buffer without PGA (data not shown). In the presence of PGA, dendritic molecule **3** disassembled to release tryptophan within approximately four days (Fig. 6), whereas dendritic molecule **4** released its tryptophan tail-units within 40 min (Fig. 7).

Under the experiment conditions the enzymatic cleavage occurs within seconds. Therefore, the observed release time of the tryptophan is also the actual disappearance time of the intermediate forms after the enzymatic cleavage. This dramatic enhancement of tail-unit release with the elimination-based system (dendritic molecule **4**) compared to the cyclization-based system (dendritic molecule **3**) is best viewed by superimposition of the graphs (Fig. 8).

We decided to apply the elimination-based dendritic system to the synthesis of an anticancer prodrug and to evaluate it in a tumor cell cytotoxicity assay. Dendritic prodrugs **15** and **16** were synthesized with the chemotherapeutic drug melphalan as a tail unit and a trigger that is activated by PGA (Fig. 9). Three equivalents of melphalan was coupled with tricarbonates **5** and **14** to afford AB_3 self-immolative dendritic prodrugs **15** and **16**, respectively.

In order to evaluate the in vitro antitumor activity of the prodrugs, compounds **15** and **16** were incubated at varied concentrations with human T-lineage acute lymphoblastic leukemia MOLT-3 cells in the presence or absence of 1 μM PGA. The data from the cell proliferation assays are presented in Figure 10. A colorometric assay based on the tetrazolium salt XTT was used to evaluate the cytotoxicity of the compounds.

Melphalan prodrugs **15** and **16** exhibited significantly (about 100-fold) reduced toxicity than free melphalan in the absence of PGA. XTT cytotoxicity assays showed a decrease of 17-fold in IC_{50} for prodrug **15** (100 vs 6 μM with PGA) and 200-fold for prodrug **16** (100 vs 0.5 μM with PGA). In the presence of PGA, prodrug **15** showed some increased cytotoxicity but still notably less than that of free melphalan (6 and 0.3 μM, respectively). However, when prodrug **16** was activated by PGA, the cytotoxicity was almost identical to that of free melphalan (IC_{50} values of free melphalan and prodrug **16** in the presence of PGA were 0.3 and 0.5 μM,

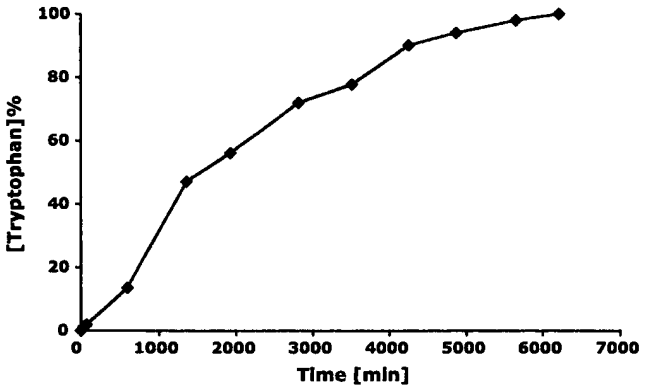

Figure 6. PGA-catalyzed release of tryptophan from dendritic compound **3** (compound **3** [500 μM] in PBS, PGA [1 mg/mL]).

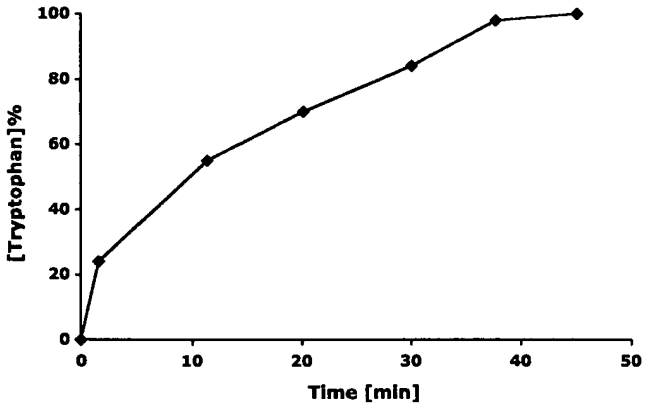

Figure 7. PGA-catalyzed release of tryptophan from dendritic compound **4** (compound **4** [500 μM] in PBS, PGA [1 mg/mL]).

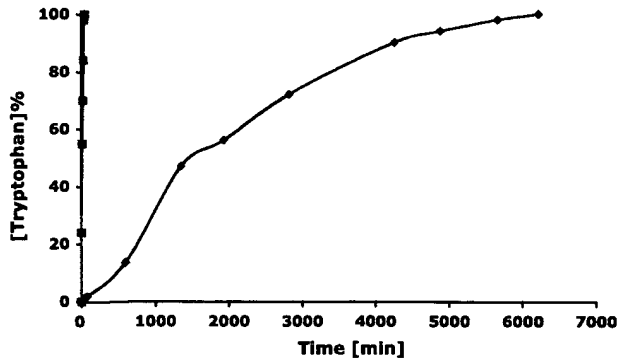

Figure 8. PGA-catalyzed release of tryptophan from dendritic compound 3 (purple) with $t_{1/2}$ of 1400 min versus release from dendritic compound 4 (red) with $t_{1/2}$ of 10 min.

respectively). Interestingly, in the absence of PGA both prodrugs were relatively not toxic even at very high doses of 100 μM. No toxicity was observed for the platform building blocks within the activity range of melphalan or melphalan prodrug.[6]

Site-specific drug delivery via a prodrug approach has generated considerable interest for enhancing the potency and for diminishing the side effects of a drug.[15] Rapid release of chemotherapeutic drugs from a prodrug system is important if a high concentration of active drug is needed at the tumor site. The described elimination-based AB_3 self-immolative dendritic prodrug system had a rapid activation pathway. Furthermore, a single cleavage by PGA was efficiently amplified to release three active drug molecules. The results shown in this study provide a proof of concept for this amplifying approach. Similar dendritic prodrugs with triggers that are activated by specific endogenous tumoral enzymes could be applied for selective chemotherapy. For example, legumain, a recently identified lysosomal protease, is a promising candidate target for prodrug therapy since it is overexpressed in the majority of human solid tumors.[16] Legumain promotes cell migration and its overexpression is associated with enhanced tissue invasion and metastasis. The enzyme cleaves the amide linkage of the tri-peptide Asn-Ala-Ala.[17] Linking this peptide, instead of the PGA substrate, to the elimination-based dendritic system described here will generate a promising self-immolative AB_3 prodrug that is suitable for site-specific drug delivery.

Additional potential application for these single-triggered dendritic systems could be in the amplification of signals in diagnostic assays. For example, chromogenic amines can be used as tail units. The amine is colorless when attached to the dendritic platform, but upon release a distinct new chromophore can be detected by spectrophotometry.[18] Introduction of phenylacetamide as a trigger will generate a molecular sensor for the enzyme PGA. A single cleavage by PGA will result in release of three reporter units and generation of a strong chromophore. There are several examples for reporter molecules with primary or secondary amine groups that can generate UV, UV–vis, or fluorescence signals.

3. Conclusions

In summary, we have designed and synthesized new AB3 self-immolative dendritic prodrug system that releases three active drugs upon a single cleavage by the model enzyme PGA. The cleavage signal is transferred from the dendron focal point to its periphery through fast elimination reactions and is amplified three-fold. The elimination-based AB_3 dendritic prodrug showed significant enhancement of drug release in comparison to a cyclization-based AB_3 dendritic prodrug. This difference was noticeably reflected in a cytotoxicity assay. Our new trimeric prodrug system could offer significant advantages in inhibition of tumor growth relative to regular monomeric prodrugs, especially if the targeted or secreted enzyme exists at relatively low levels in the malignant tissue.

4. Experimental

4.1. General

All reactions requiring anhydrous conditions were performed under an Ar or N_2 atmosphere. Chemicals and solvents were either A.R. grade or purified by standard techniques. Thin layer chromatography (TLC) was performed on silica gel plates Merck 60 F_{254}; compounds

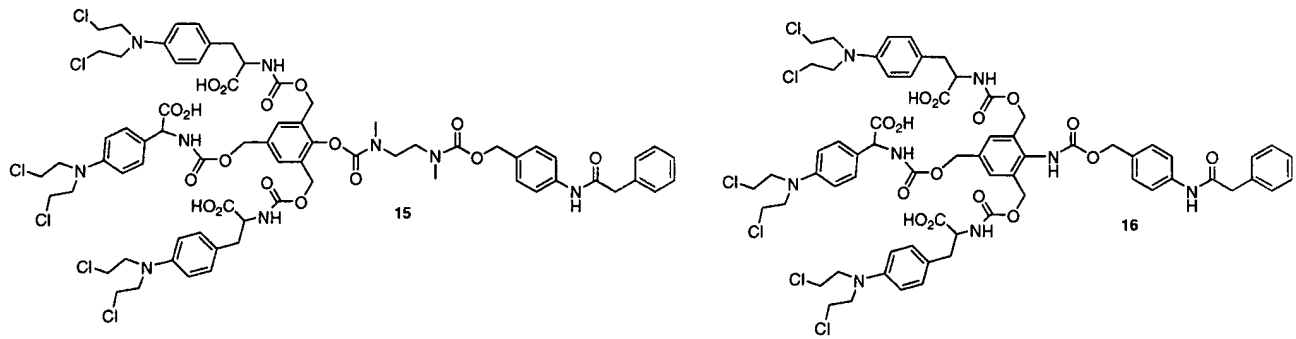

Figure 9. Chemical structures of AB3 self-immolative dendritic prodrugs with melphalan tail units and a trigger that is activated by PGA.

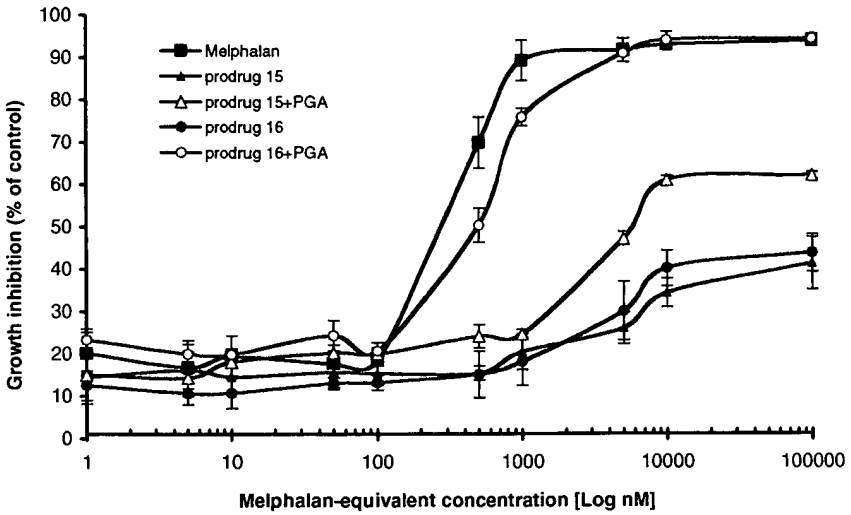

Figure 10. Growth inhibition assay of leukemia MOLT-3 cell line with dendritic prodrug **15** or **16** in the presence or absence of PGA. Cells were incubated for 72 h. Full blue squares represent melphalan, full green triangles prodrug **15**, empty green triangles prodrug **15** with PGA, full red circles prodrug **16**, empty red circles prodrug **16** with PGA. Symbols represent mean ± SD.

were visualized by irradiation with UV light and/or by treatment with a solution of phosphomolybdic acid (25 g), $Ce(SO_4)_2 \cdot H_2O$ (10 g), concd H_2SO_4 (60 mL), and H_2O (940 mL), followed by heating. Flash chromatography was performed by using silica gel Merck 60 (particle size 0.040–0.063 mm) and the eluent given in parentheses. ^1H NMR spectroscopy was performed by using a Bruker AMX 200 or 400 instrument. The chemical shifts are expressed in δ relative to tetramethylsilane (TMS) ($\delta = 0$ ppm) and the coupling constants J in Hz. The spectra were recorded in $CDCl_3$ or CD_3OD as a solvent at room temp. HR-MS: liquid secondary ionization (LSI-MS): VG ZAB-ZSE with 3-nitrobenzyl-alcohol matrix. All reagents, including salts and solvents, were purchased from Sigma–Aldrich (Milwaukee, MN).

4.2. Cell culture

Tumor cells (Molt-3 human acute lymphoblastic leukemia cells) were a kind gift from Holger Lode (Charlté Children's Hospital, Berlin). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) at 37 °C with 5% CO_2.

4.3. Compound 3

Compound **5** (20 mg, 0.019 mmol) was dissolved in DMF. Tryptophan (15 mg, 0.076 mmol) was added, followed by the addition of Et_3N (150 μL, 1 mmol). The reaction mixture was stirred overnight and monitored by TLC (EtOAc/MeOH 9:1 + 1% of acetic acid) and by HPLC. After completion, the solvent was removed under reduced pressure and the crude product was purified by column chromatography on silica gel (EtOAc/MeOH 9:1, 1% of acetic acid) followed by additional purification using a reverse phase chromatography (HPLC) with C-18 semi-preparative column, to give compound **3** (23 mg, 75%) in the form of a white powder. HRMS (MALDI-TOF): *m/z* calcd for $C_{66}H_{65}N_9O_{17}Na$: 1278.4355; found: 1278.4390 $[M+Na]^+$.

4.4. Compound 7

Compound **6**[19] (2.95 g, 17.53 mmol) was dissolved in DMF and cooled to 0 °C. Imidazole (4.77, 70.16 mmol) and TBSCl (10.57 g, 70.16 mmol) were added. The reaction was allowed to warm to room temperature and was stirred for additional 3 h. The reaction was monitored by TLC (EtOAc/Hex 5:95). After completion, the reaction was diluted with EtOAc and washed with NH_4Cl solution. The organic layer was dried over magnesium sulfate and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (EtOAc/Hex 5:95) to give compound **7** (6.09 g, 68%) in the form of colorless oil.

^1H NMR (200 MHz, $CDCl_3$): $\delta = 7.12$ (3H, s); 4.69 (6H, s); 0.92 (27H, s); 0.06 (18H, s). ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 143.27$, 124.34, 67.00, 27.94, 20.39, −3.28. MS (FAB): *m/z*: 509.4 $[M+H]^+$.

4.5. Compound 8

Acetic anhydride (30 mL) was cooled to 5 °C and nitric acid (2 mL, 71%) was added dropwise. After the addition was completed, the mixture was stirred for 15 min at room temperature and then cooled to −20 °C. A solution of compound **7** (5.85 g, 11.46 mmol) in 10 mL of acetic anhydride was added dropwise. The reaction mixture was allowed to warm to 0 °C and was stirred for additional 30 min. After completion, the reaction was diluted with EtOAc and was washed with $NaHCO_3$ solution followed by brine. The organic layer was dried over magnesium sulfate and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (EtOAC/Hex 5:95) to give compound **8** (4.78 g, 75%) in the form of a yellow oil.

^1H NMR (200 MHz, $CDCl_3$): $\delta = 7.49$ (2H, s); 4.75 (6H, s); 0.92 (27H, s); 0.06 (18H, s). ^{13}C NMR (100 MHz,

$CDCl_3$): δ = 144.64, 141.23, 122.30, 64.97, 61.39, 25.93, 18.36, −5.29. MS (FAB): *m/z*: 556.4 $[M+H]^+$.

4.6. Compound 9

Compound **8** (5.43 g, 9.76 mmol) was dissolved in a 50:50 THF/MeOH solution. A catalytic amount of palladium was added to the mixture, followed by the addition of ammonium formate (1 g, 15.8 mmol). The reaction mixture was stirred in room temperature for 2.5 h and monitored by TLC (EtOAc/Hex 5:95). After completion, the salts were filtered out and the solvent was removed under reduced pressure. The residue was diluted with EtOAc and washed with brine. The organic layer was dried over magnesium sulfate, and the crude product was purified by column chromatography on silica gel (EtOAc/Hex 5:95) to give compound **9** (4.39 g, 85%) in the form of a yellow oil.

^{1}H NMR (200 MHz, $CDCl_3$): δ = 6.9 (2H, s); 4.66 (6H, s); 4.56 (2H, s); 0.87 (27H, s); 0.03 (18H, s). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 146.93, 131.40, 128.51, 126.96, 67.02, 66.88, 27.79, 20.15, −3.26. MS (FAB): *m/z*: 525.3 $[M+H]^+$.

4.7. Compound 12

Toluene was heated to reflux (110 °C) and a solution of 20% phosgene in toluene (9.8 mL, 19 mmol) was added. Then, a solution of compound **9** (1 g, 1.9 mmol) in toluene was slowly added dropwise with a syringe. The reaction mixture was stirred for 30 min at reflux and monitored by ^{1}H NMR. After the isocyanate derivative was observed, the solvent was removed under reduced pressure. A solution of compound **11**[14] (596 mg, 2.47 mmol)in DMF, followed by 0.5 mL Et_3N, was added to the isocyanate residue. The reaction mixture was stirred for 1 h and monitored by TLC (EtOAc/Hex 30:70). After completion, the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (EtOAc/Hex 20:80) to give compound **12** (751 mg, 50%) in the form of a white solid.

^{1}H NMR (200 MHz, $CDCl_3$): δ = 7.41–7.29 (11H, m); 5.11 (2H, s); 4.71 (2H, s); 4.65 (2H, s); 3.74 (2H, s); 0.94 (27H, s); 0.08 (18H, s). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 170.96, 156.20, 139.49, 138.00, 136.29, 131.44, 131.19, 130.79, 129.65, 126.69, 124.34, 121.59, 68.46, 66.70, 64.87, 46.79, 27.92, 20.21, −3.42. MS (FAB): *m/z*: 525.3 $[M+H]^+$.

4.8. Compound 13

Compound **12** (286 mg, 0.36 mmol) was dissolved in MeOH/DCM 1:1 and Amberlyst 15 was added. The reaction mixture was stirred in room temperature for 2 h and monitored by TLC (EtOAc/Hex 9:1). After completion, the Amberlyst 15 was filtered out and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (EtOAc/Hex 9:1) to give compound **13** (105 mg, 65%) in the form of a white solid.

^{1}H NMR (200 MHz, MeOD): δ = 7.53–7.29 (11H, m); 5.09 (2H, s); 4.58 (2H, s); 4.54 (4H, s); 3.64 (2H, s). ^{13}C NMR (400 MHz, MeOD): δ = 170.86, 155.88, 140.33, 138.34, 135.28, 128.61, 128.08, 126.45, 124.73, 119.62, 66.18, 63.60, 63.43, 60.05, 29.22. MS (FAB): *m/z*: 451.2 $[M+H]^+$.

4.9. Compound 14

Compound **13** (236 mg, 0.523 mmol) was dissolved in dry THF and a catalytic amount of pyridine was added. The solution was cooled to −20 °C and a solution of PNP-chloroformate (1.6 g, 7.8 mmol) in dry THF was added dropwise. The temperature was not allowed to exceed −10 °C. The mixture was stirred for 6 h and monitored by HPLC and by TLC (EtOAc/Hex 50:50). After completion, the reaction mixture was diluted with EtOAc and washed with saturated NH_4Cl solution. The organic layer was dried over magnesium sulfate and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (EtOAc/Hex 50:50) to give compound **14** (395 mg, 80%) in the form of a pale yellow powder.

^{1}H NMR (200 MHz, $CDCl_3$): δ = 8.26 (6H, m); 7.65 (2H, s); 7.52–7.33 (15H, m); 5.36 (4H, s); 5.27 (2H, s); 5.14 (2H, s); 3.70 (2H, s). ^{13}C NMR (400 MHz, $CDCl_3$): δ = 175.54, 160.46, 160.32, 157.56, 150.53, 143.45, 139.91, 138.37, 136.61, 134.21, 133.79, 132.21, 130.45, 128.91, 124.99, 75.29, 72.87, 72.34, 72.03, 49.05, 35.2. HRMS (MALDI-TOF): *m/z* calcd for $C_{46}H_{35}N_5O_{18}Na$: 968.1846; found: 968.1869 $[M+Na]^+$.

4.10. Compound 4

Compound **14** (20 mg, 0.021 mmol) was dissolved in DMF. Tryptophan (17.3 mg, 0.085 mmol) was added, followed by the addition of Et_3N (15 μL, 0.1 mmol). The reaction mixture was stirred overnight and monitored by TLC (EtOAc/MeOH 9:1 + 1% of acetic acid) and by HPLC. After completion, the solvent was removed under reduced pressure and the crude product was purified by column chromatography on silica gel (EtOAc/MeOH 9:1, 1% of acetic acid) followed by additional purification using a reverse phase HPLC with C-18 semi-preparative column (H_2O/CH_3CN gradient 10%–100%), to give compound **4** (18 mg, 75%) in the form of a white powder. HRMS (MALDI-TOF): *m/z* calcd for $C_{61}H_{56}N_8O_{15}Na$: 1163.3647; found: 1163.3757 $[M+Na]^+$.

4.11. Compound 15

Compound **5** (20 mg, 0.019 mmol) was dissolved in DMF. Melphalan (15.5 mg, 0.076 mmol) was added, followed by the addition of Et_3N (15 μL, 0.1 mmol). The reaction mixture was stirred overnight and monitored by TLC (EtOAc/MeOH 9:1 + 1% of acetic acid) and by HPLC. After completion, the solvent was removed under reduced pressure and the crude product was purified by column chromatography on silica gel (EtOAc/MeOH 9:1, 1% of acetic acid) followed by additional purification using a reverse phase HPLC with C-18 semi-preparative column (H_2O/CH_3CN gradient

10%–100%), to give compound **15** (20 mg, 70%) in the form of a white powder. HRMS (MALDI-TOF): *m/z* calcd for $C_{72}H_{83}N_9O_{17}Cl_6Na$: 1578.3945; found: 1578.3930 $[M+Na]^+$.

4.12. Compound 16

Compound **14** (20 mg, 0.021 mmol) was dissolved in DMF. Melphalan (17.3 mg, 0.085 mmol) was added, followed by the addition of Et_3N (15 μL, 0.1 mmol). The reaction mixture was stirred over night and monitored by TLC (EtOAc/MeOH 9:1 + 1% of acetic acid) and by HPLC. After completion, the solvent was removed under reduced pressure and the crude product was purified by column chromatography on silica gel (EtOAc/MeOH 9:1, 1% of acetic acid) followed by additional purification using a reverse phase chromatography (HPLC) with C-18 semi-preparative column (H_2O/CH_3CN gradient 10%–100%), to give compound **16** (21 mg, 70%) in the form of a white powder. HRMS (MALDI-TOF): *m/z* calcd for $C_{67}H_{74}N_8O_{15}Cl_6Na$: 1463.3262; found: 1463.3297 $[M+Na]^+$.

4.13. Cell proliferation assay with XTT reagent

Molt-3 cells were harvested from culture flasks, resuspended in cell culture medium, and plated at a density of 5×10^3 cells/well in 100 μL onto 96-well culture plate in RPMI 1640 medium supplemented with 10% FBS. Cells were challenged with prodrug 15 or prodrug 16 (1–100,000 nM) in the presence or absence of PGA enzyme (1 μM) and incubated for 72 h (5% CO_2). Control cells were grown with 10% FBS.

Activation solution (100 μL) was added to 5 mL XTT reagent. The reaction solutions (50 μL) were added to each well. The plate was incubated for 2 h, shaken gently to evenly distribute the dye in the wells. Absorbance was measured at a wavelength of 450–500 nm. In order to measure reference absorbance (to measure nonspecific readings), a wavelength of 630–690 nm was used.

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